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# A Membrane Cytoskeleton from *Dictyostelium discoideum*

## I. Identification and Partial Characterization of an Actin-binding Activity

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**ABSTRACT** *Dictyostelium discoideum* plasma membranes isolated by each of three procedures bind F-actin. The interactions between these membranes and actin are examined by a novel application of falling ball viscometry. Treating the membranes as multivalent actin-binding particles analogous to divalent actin-gelation factors, we observe large increases in viscosity (actin cross-linking) when membranes depleted of actin and myosin are incubated with rabbit skeletal muscle F-actin. Pre-extraction of peripheral membrane proteins with chaotropes or the inclusion of Triton X-100 during the assay does not appreciably diminish this actin cross-linking activity. Lipid vesicles, heat-denatured membranes, proteolyzed membranes, or membranes containing endogenous actin show minimal actin cross-linking activity. Heat-denatured, but not proteolyzed, membranes regain activity when assayed in the presence of Triton X-100. Thus, integral membrane proteins appear to be responsible for some or all of the actin cross-linking activity of *D. discoideum* membranes.

In the absence of MgATP, Triton X-100 extraction of isolated *D. discoideum* membranes results in a Triton-insoluble residue composed of actin, myosin, and associated membrane proteins. The inclusion of MgATP before and during Triton extraction greatly diminishes the amount of protein in the Triton-insoluble residue without appreciably altering its composition. Our results suggest the existence of a protein complex stabilized by actin and/or myosin (membrane cytoskeleton) associated with the *D. discoideum* plasma membrane.

Interactions of cytoskeletal structures with eukaryotic cell membranes are thought to be important for maintenance of cellular shape, endo- and exocytosis, movement, and cell-cell and cell-substrate attachment (1, 2, 45, 74, 79, 81, 86). Although many membrane-cytoskeleton interactions may be transient, many are stable enough to allow the isolation of membranes with part of the cytoskeleton still attached (14, 45, 58, 78, 86). Actin is one of the most frequently identified cytoskeletal proteins in such preparations (86). Much is known about the interactions between actin and soluble cytoplasmic proteins (40, 79), but less is known about the interactions between actin and membranes.

Amoebae of the cellular slime mold, *Dictyostelium discoi-*

*deum*, provide an excellent system for investigating the dynamics of cytoskeleton-membrane interactions. A number of cytoskeletal and contractile proteins from these cells have been identified and characterized (11, 13, 17, 32, 68), and numerous membrane isolation techniques have been described (16, 26, 28, 37, 49, 55, 59, 62, 68). *D. discoideum* amoebae exhibit endo- and exocytosis, amoeboid movement, cell-cell interactions, and cell-substrate attachments (14, 42, 79, 80). Also, mutants of motile functions have been identified (12). This report identifies an actin-binding activity associated with the *D. discoideum* membrane cytoskeleton and, thus, initiates the characterization of cytoskeletal protein-membrane interactions in the amoeboid stage of *D. discoideum*.

## MATERIALS AND METHODS

### Chemicals

ATP was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). PIPES, Tris, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), diisopropylfluorophosphate (DFP), Triton X-100, *N*-carbobenzoxypheylalanine, dipalmitoylphosphatidylcholine (DPPC), 1,10-phenanthroline monohydrate, and protease from *Streptomyces griseus*, type VI (pronase P) were supplied by Sigma Chemical Co. (St. Louis, Mo.). Ultra pure urea was obtained from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.). SDS and other reagents used for SDS polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, Calif.). Three times crystallized concanavalin A (Con A) was obtained from Miles-Yeda Ltd., Israel. Molecular weight standards were from Pharmacia Fine Chemicals, Div., Pharmacia, Inc. (Piscataway, N. J.). Ampules of osmium tetroxide and glutaraldehyde solutions under nitrogen were obtained from Polysciences, Inc. (Warrington, Pa.). Spectraanalyzed methanol was supplied by Fisher Scientific Co. (Medford, Mass.), and spectrophotometric grade chloroform by Aldrich Chemical Co., Inc. (Metuchen, N. J.). Water was deionized after distillation. All other chemicals were reagent grade.

Human erythrocyte membranes and purified spectrin were kindly supplied by Dr. W. R. Hargreaves. Twice recrystallized cholic acid was the generous gift of Dr. J. Tyler.

### Electron Microscopy

Samples were prepared for electron microscopy by fixation at 0°C for 30 min in 3% glutaraldehyde in 20 mM sodium phosphate, pH 6.8. Samples were postfixed in 2% osmium tetroxide in 20 mM sodium phosphate, pH 6.8, for 90 min at 0°C. Some samples were stained en bloc for 40 min at 0°C with 1% aqueous uranyl acetate. Samples were washed twice in water, dehydrated in acetone, and embedded in Spurr resin ("A" recipe). Thin sections were stained with 2% aqueous uranyl acetate and lead citrate, and examined at 80 kV in a Philips 301 electron microscope.

The fixation and staining procedures used here are similar to those used in a number of papers reporting the purification of *D. discoideum* plasma membranes (18, 26, 28, 49, 66). These procedures, particularly the prolonged postfixation in osmium tetroxide, preserve the appearance of membranous structures at the expense of microfilaments and related cytoskeletal assemblies (57). In particular, the plasma membranes of these cells, fixed and stained as described, assume a characteristic, dark, bold profile which, at high magnification, is visualized as a well-demarcated, trilaminar structure (18, 26, 28, 49, 66).

### Viscosity Measurements

Viscosity was measured using a low-shear falling ball viscometer (29, 46) as previously described (24), under conditions optimal for gelation of actin by *D. discoideum* cell extracts (32). These assay conditions were 50 mM KCl, 20 mM PIPES, pH 6.8, 1 mM MgATP, 2.0 mM EGTA, and 0.1 mM CaCl<sub>2</sub> (assay buffer). Membranes and actin contributed an additional 2–10 mM sodium phosphate and <0.3 mM each of sodium azide, DTT, and Tris-HCl (concentrations held constant during each experiment). Unless otherwise indicated, rabbit skeletal muscle G-actin was added to the assay mixture at 0°C immediately after addition of membranes. Samples were incubated at 28°C for 1 h as described in the accompanying paper (25).

### Assays

Protein was determined by the procedure of Lowry et al. (43) with bovine serum albumin as a standard. Assays of membrane protein were performed in the presence of 1% SDS.

Organic solvent-extractable phosphorus was measured as an estimate of phospholipid phosphorus. 50- $\mu$ l to 1-ml aliquots of membranes containing up to 2 mg of membrane protein were extracted with water-methanol-chloroform according to the procedure of Bligh and Dyer (7, 38). The chloroform layer from the final centrifugation was assayed for total phosphorus by the method of Ames (3) with DPPC as a standard.

Alkaline phosphatase, a presumed marker for *D. discoideum* plasma membranes (26, 28, 55, 62), and cytochrome *c* oxidase, a classical marker for inner mitochondrial membranes (4), were assayed as described by Hodges and Leonard (33). Inorganic phosphate released in the alkaline phosphatase assay was measured by the method of Ames (3).

### Polyacrylamide-Gel Electrophoresis

Gradient SDS slab gels were prepared and run according to the method of Laemmli (41). Samples were dissolved and reduced for 5–10 min at 70°C with

sample buffer (1% SDS, 10% sucrose, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 32 mM DTT, 0.1% bromphenol blue, 0.1% pyronin Y). Gels were stained with Coomassie Blue and destained as described by Fairbanks et al. (23). Apparent molecular weights of *D. discoideum* membrane polypeptides were determined using standard curves derived from the relative electrophoretic mobilities of the following polypeptides (numbers in parentheses refer to polypeptide molecular weights in kilodaltons): thyroglobulin (330); spectrin, band 1<sup>1</sup> (240); spectrin, band 2 (220); ferritin (220, 18.5); phosphorylase a (94); human erythrocyte band 4.1 (86); human erythrocyte band 4.2 (78); albumin (67); catalase (60); ovalbumin (43); lactate dehydrogenase (36); carbonic anhydrase (30); Con A (27); soybean trypsin inhibitor (20.1); myoglobin (16.9); hemoglobin (16);  $\alpha$ -lactalbumin (14.4); cytochrome *c* (12.4).

### Peptide Mapping

Two-dimensional tryptic peptide mapping of <sup>125</sup>I-labeled polypeptides was performed as described by Elder et al. (22) and as later modified (39, 44). The first-dimensional electrophoresis was carried out for 80 min at 0.5°C. Subsequent chromatography and all other steps in this procedure were performed as described (39, 44).

### Preparation of Actin and Membranes

**ACTIN:** G-actin was prepared from an acetone powder of rabbit skeletal muscle with a single cycle of polymerization and sedimentation from 0.8 M KCl (69) and was depolymerized and stored as described in the accompanying paper (25).

**MEMBRANES:** Amoebae of *D. discoideum* (strain A3) were harvested from axenic culture while in late exponential growth phase at a concentration of  $\sim 1 \times 10^7$  cells/ml. The cells were collected by centrifugation at 650 g for 2 min and were washed twice with Sorenson's phosphate buffer (17), pH 6.1. Unless otherwise specified, membranes were prepared by the following modification of the method of Spudich and co-workers (68, 70, 84). The cells were mixed 1:1 with lysis buffer (10 mM Tris-HCl, pH 7.6, 30% sucrose, 0.4 mM DTT, 40 mM sodium pyrophosphate, 0.02% sodium azide, 0.1 mg/ml PMSF, 2 mM EDTA, 5 mM 1,10-phenanthroline, 2 mM *N*-carbobenzoxypheylalanine, 0.5% ethanol) and lysed at 0°C either by homogenization with a Dounce homogenizer (Kontes Co., Vineland, N. J.) or by explosive decompression (1,100 lb/in<sup>2</sup>, 25 min) with a Parr bomb (Parr Instrument Co., Moline, Ill.). Results similar to those described below were also obtained with membranes from cells disrupted by freezing in liquid nitrogen (70) or by forced passage through a 5- $\mu$ m Nuclepore filter (Nuclepore Corp., Pleasanton, Calif.) (59). After the cells were broken, the suspension was diluted with lysis buffer and membranes were collected by centrifugation at 39,000 g for 15 min at 2°C. The flocculent top layer of the pellet was transferred to a clean centrifuge tube and washed once with a large excess of lysis buffer. After each centrifugation, the cloudy supernate and the tightly packed bottom layer of the pellet were discarded. The membranes were resuspended in either lysis buffer or 20 mM sodium phosphate, pH 6.8, and layered onto a discontinuous sucrose gradient (35/55% sucrose, with either 20 mM sodium phosphate, pH 6.8, or 100 mM KCl, 10 mM Tris-HCl, pH 7.6, throughout). After a 1-h centrifugation at 131,000 g, the material banding at the 35/55% boundary was collected and washed twice with 20 mM sodium phosphate, pH 6.8, at 27,000 g for 10 min at 2°C. Membranes were resuspended with 20 mM sodium phosphate, pH 6.8, 0.02% sodium azide and stored at 0°C. Although membranes were generally used within 2 wk of preparation, experiments performed with membranes stored as long as 4 mo indicated little or no loss of actin cross-linking activity.

**TRITON-EXTRACTED MEMBRANES:** Membranes at a final concentration of 5–8 mg of membrane protein/ml were extracted at 0°C for 10 min with 20 mM sodium phosphate, pH 6.8, 1 mM DTT, 3% Triton X-100 (final concentrations). Unless otherwise indicated, the Triton-insoluble residues were collected by centrifugation through 35% sucrose, 20 mM sodium phosphate, pH 6.8, at 39,000 g for 20 min at 2°C. Immediately after preparation, the sticky pellets were either fixed for electron microscopy or resuspended with 20 mM sodium phosphate, pH 6.8, 0.02% sodium azide. Clumping was disrupted with a few strokes of a tissue grinder with a Teflon pestle (Arthur H. Thomas Co., Philadelphia, Penn.). Approximately 35% of the total membrane protein and 5% of the total membrane phospholipid were recovered with the resuspended pellet as was a small amount of nucleic acid (2.5–4.0% by weight based on ratios of 280:260 OD [54]).

**LOW IONIC STRENGTH-EXTRACTED MEMBRANES:** To remove actin, myosin, and other peripheral membrane proteins, *D. discoideum* membranes in 3.5–7.0 ml of 20 mM sodium phosphate, pH 6.8, were diluted to  $\sim 40$  ml with water and collected by centrifugation at 39,000 g for 20 min at 2°C. The loose pellet was diluted 1:1 with water and transferred to a dialysis bag. After overnight

<sup>1</sup> Nomenclature of erythrocyte membrane proteins is according to Steck (72).

dialysis at 0°C vs. 150 vol of 0.1 mM EDTA, 0.2 mM sodium phosphate, pH 7.6, the membranes were diluted to ~40 ml with dialysis buffer and centrifuged at 39,000 g for 60 min at 2°C. The pellet was washed once with 20 mM sodium phosphate, pH 6.8, 0.02% sodium azide, resuspended, and stored at 0°C in this buffer. Clumps of the pelleted membranes were disrupted by gentle homogenization. About 50% of the total membrane protein and ~82% of the total membrane phospholipid were recovered after this procedure.

**KCL, UREA-EXTRACTED MEMBRANES:** To remove additional peripheral membrane proteins, low ionic strength-extracted membranes in 20 mM sodium phosphate, pH 6.8, at a concentration of 6–10 mg of membrane protein/ml were extracted at 0°C for 45 min with an equal volume of 2 M KCl, 7 M urea, 0.1 M glycine, 20 mM EDTA, 50 µg/ml PMSF, pH 7.2. Extracted membranes were pelleted by centrifugation at 149,000 g for 60 min at 2°C. The pellet was resuspended in a small volume of 20 mM sodium phosphate, pH 6.8, and dialyzed against 1,000 vol of this buffer for 1–2 d with at least one buffer change. After this stripping procedure, an average of 65% of the low ionic strength-extracted membrane protein and 110% of these membranes' phospholipid were recovered.

**NAOH-EXTRACTED MEMBRANES:** Low ionic strength-extracted membranes or KCl, urea-extracted membranes in 20 mM sodium phosphate, pH 6.8, were further extracted with sodium hydroxide to remove nearly all peripheral membrane proteins (73). The following solutions were mixed, in order, in a 30-ml Corex (E. I. duPont de Nemours & Co., Wilmington, Del.) centrifuge tube: 16.0 ml of water, 1.9 ml of 1 N NaOH (Fisher Scientific Co., certified solution), 0.2 ml of 100 mM DTT, and 0.9 ml of membranes in buffer (final pH ~12.5). After a 60-min incubation at 0°C, the mixture was sonicated with two 5-s bursts from a Branson sonicator (Branson Sonic Power Co., Danbury, Conn.) at 25–30 W. The NaOH-extracted membranes were collected in a Beckman SW40 tube (Beckman Instruments, Inc., Fullerton, Calif.) by centrifugation at 284,000 g for 60 min at 2°C through a shelf consisting of 2% sucrose, 20 mM sodium phosphate, pH 6.8. An average of 6% of the KCl, urea-extracted membrane protein and 35% of the phospholipid were recovered in the nearly white pellet.

**PROTEASE TREATMENT OF MEMBRANES:** Proteolytic digestion of KCl, urea-extracted membranes was carried out at 37°C in a 2-ml reaction mixture containing 5–10 mg of membrane protein, 0.8 mg of pronase P (Sigma Chemical Co.), and 20 mM sodium phosphate, pH 6.8. After 30 min, 200 µl of 100 mM EDTA, pH 7.6, was added and the incubation was continued for another 90 min. The reaction was stopped by the addition of 4 ml of 16 mM DFP, 100 µl of 30 mg/ml PMSF in ethanol, and 3.7 ml of 20 mM sodium phosphate, pH 6.8. After 15 min at 37°C in the presence of the protease inhibitors, the membranes were centrifuged at 378,000 g for 90 min at 2°C. The pellet was resuspended to 10 ml with 6.4 mM DFP, 300 µg/ml PMSF, 20 mM sodium phosphate, pH 6.8, 1% ethanol, incubated a second time at 37°C for 15 min, and centrifuged as before. The final pellet was resuspended to ~1 ml with 1.6 mM DFP, 300 µg/ml PMSF, 0.02% sodium azide, 20 mM sodium phosphate, pH 6.8. About 32% of the KCl, urea-extracted membrane protein and 99% of the phospholipid were recovered. Control membranes were treated identically except that protease was omitted from the initial incubation.

## Preparation of Lipid Vesicles

**DPPE VESICLES:** DPPE vesicles were generated by the ether-injection technique of Deamer and Bangham (19). 8 ml of 4:1 diethyl ether:methanol containing 20 mg DPPE were injected at the rate of 0.25 ml/min into 8 ml of 20 mM sodium phosphate, pH 6.8, at 68°C. The vesicles were collected by centrifugation at 252,000 g for 10 min at 4°C and washed three times in large volumes of the above buffer.

**D. DISCOIDEUM LIPID VESICLES:** Total lipid was extracted from isolated *D. discoideum* membranes using the procedure of Bligh and Dyer (7, 38). Organic solvent was evaporated, first with a stream of nitrogen at 28°C and then by prolonged vacuum desiccation. The dried lipids were solubilized by adding 0.5 ml 10% sodium cholate, pH 8.7, warming to 60°C, and sonicating with two 30-s bursts at 25 W with a Branson sonicator. Vesicles from *D. discoideum* lipids were formed upon dialysis of the detergent suspension for 2 d against 1,000 vol of 20 mM sodium phosphate, pH 6.8, with four buffer changes.

## RESULTS

### Major Polypeptides of *D. discoideum* Membranes

The SDS polyacrylamide gel pattern of membranes from vegetative cells (Fig. 1a, lane M) is similar to the gel pattern of membranes from developing cells (Fig. 1b, lane M) and to gel patterns obtained by other investigators for plasma mem-

brane-enriched fractions from *D. discoideum* (34, 52, 66). A darkly staining doublet at ~30,000 daltons (bands 6 and 6.2 in Fig. 1) and an intense band at ~72,000 daltons (band 3 in Fig. 1) are prominent in all these *D. discoideum* membrane preparations (Fig. 1 and references 34, 52, and 66). Band-by-band cross-identification of other polypeptides is complicated by a number of factors including the large number of bands, reported differences in molecular weights for what may be the same polypeptides, selective removal of peripheral membrane proteins, and different amounts of proteolysis.

Little or no proteolysis is evident in *D. discoideum* membranes prepared by our method. The presence of 0.2 mM DFP before and during cell breakage does not detectably change the membranes' SDS polyacrylamide gel pattern. Furthermore, no two tryptic peptide maps of the membrane polypeptides numbered in Fig. 1a are sufficiently similar to suggest a precursor-proteolytic product relationship (data not shown). Omission during membrane isolation of the protease inhibitors listed in Materials and Methods does alter the gel pattern (data not shown). Tryptic peptide mapping of additional bands present in these latter preparations identifies a polypeptide with an apparent mol wt of 200,000 daltons that is probably a proteolytic fragment of the 230,000-dalton polypeptide (band 1.1 in Fig. 1). Similarly, tryptic peptide mapping of a 70,000-dalton polypeptide observed in proteolyzed membranes suggests a precursor-proteolytic product relationship between a 72,000-dalton polypeptide (band 3 in Fig. 1) and the 70,000-dalton polypeptide.

To simplify comparisons of gel patterns, we number sequentially from the slowest to the fastest mobility the major Coomassie Blue-staining bands of *D. discoideum* in a fashion analogous to that used for erythrocyte membrane polypeptides (23, 71). Arabic numerals are used so that other bands can be designated as decimal fractions in this and future studies.

### Triton Extraction of *D. discoideum* Membranes

Although some biological membranes are completely solubilized by nonionic detergents, Triton X-100 extraction of many intact cells or isolated membranes results in insoluble residues that retain some of the shape and much of the electron microscope appearance of the membranes from which they derive (6, 31, 63, 70, 77, 88). While the cellular functions of most Triton-insoluble residues are poorly understood, many are believed to play a cytoskeletal role in the intact cell (6, 31, 64).

Triton extraction of isolated *D. discoideum* membranes and subsequent centrifugation on a 10–55% sucrose gradient separate the membrane polypeptides into three fractions (Fig. 1a). One set of polypeptides fails to enter the gradient (Fig. 1a, lane S), another group sediments into the top portion of the gradient (Fig. 1a, lane 10%), and the third set forms a pellet (Fig. 1a, lane P). The first two of these three groups have not been further investigated and are jointly referred to as the Triton supernate. The third group of polypeptides, the Triton pellet, cosediments under a variety of centrifugation conditions and through concentrations of sucrose as high as 64% ( $d_{4}^{20} = 1.30$ ).

When *D. discoideum* membranes are extracted with Triton X-100 in 20 mM sodium phosphate, pH 6.8, most of the membrane-associated actin and myosin sediment with the Triton pellet (Fig. 1a and b). However, the addition of ATP (data not shown) or MgATP (reagents that temporarily reverse the association of myosin with actin [76]) to the membranes before

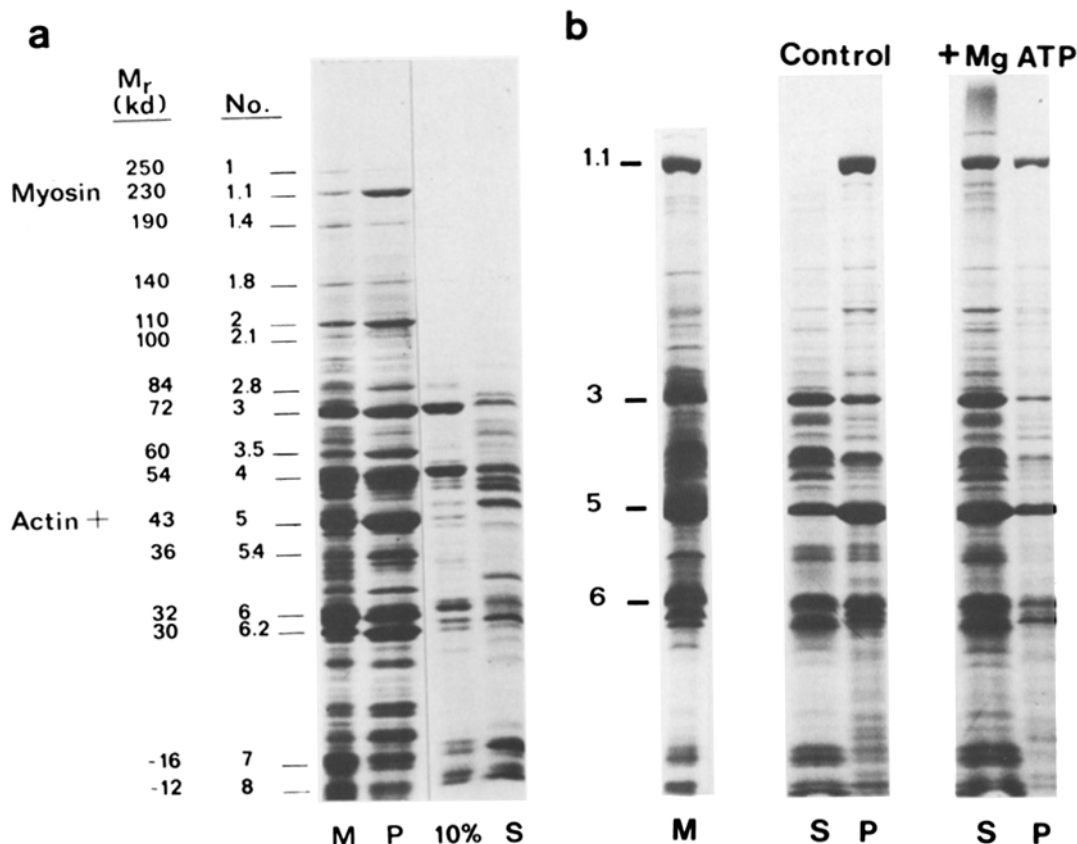


FIGURE 1 (a) 6–16% SDS polyacrylamide slab gels of membranes from vegetative amoebae before (M) and after Triton extraction and fractionation of membranes on a 10–55% sucrose gradient. After centrifugation at 230,000 g for 60 min at 2°C, fractions were collected from the bottom of the tube. Equal volumes of the resuspended Triton pellet (P), the fraction containing 10% sucrose (10%), and the Triton supernate (S) were loaded on the gel. Very little protein was observed elsewhere in the gradient. Approximately 40 µg of protein was loaded onto lanes M and P. Also presented are the apparent molecular weights ( $M_r$ ) in kilodaltons and an arbitrary numerical nomenclature (No.) for the prominent Coomassie Blue-stained bands. (b) 6–16% SDS polyacrylamide slab gels of membranes from developing cells before (M) and after Triton extraction and centrifugation through 35% sucrose cushions. Amoebae were allowed to develop for 5 h at 21°C in Sorenson's phosphate buffer, pH 6.1, before isolation of membranes. Control membranes were extracted as described in Materials and Methods; membranes plus MgATP were extracted similarly except that 10 mM MgATP was included in the extraction solution and 0.1 mM MgATP was included in the sucrose cushion. Equal volumes of the Triton supernates (S) and the Triton pellets resuspended to the initial volume (P) were loaded on the slab gel. Similar results were obtained with membranes derived from vegetative cells. After 1–4 d of storage, the membranes became insensitive to the presence of MgATP. The inclusion in the Triton extraction solution of 0.17 µM colcemid, 1 mM CaCl<sub>2</sub>, 1 mM EGTA, 1.5 µg/ml RNase, 0.75 mg/ml PMSF, 10 mM DTT, 50 µM cytochalasin B, or 1.0 µM cAMP had no noticeable effect on the amount or composition of the pellet; inclusion of 10 mM MnCl<sub>2</sub> or MgCl<sub>2</sub> increased the amount of precipitable protein. In this and the following figures, numbered bands correspond to those designated by the numerical nomenclature of Fig. 1 a.

and during extraction with Triton X-100 releases into the supernate not only actin and myosin, but also most of the other major Coomassie Blue-staining bands (Fig. 1 b).

#### Identification of Actin and Myosin in *D. discoideum* Membrane Preparations

Comparison of peptide maps of the membrane polypeptides numbered in Fig. 1 with peptide maps of polypeptides prominent in contracted pellets prepared from *D. discoideum* cell extracts (32) has identified two cytoplasmic contractile proteins which are also associated with isolated *D. discoideum* membranes. These two polypeptides, designated as bands 1.1 and 5 in Fig. 1, comigrate on SDS polyacrylamide gels with *D. discoideum* myosin and actin, respectively, and give rise to peptide maps that are strikingly similar to those derived from

cytoplasmic myosin and actin (Fig. 2).<sup>2</sup> The peptide map of band 5 also contains numerous spots not found in the peptide map of cytoplasmic *D. discoideum* actin, suggesting the presence of one or more structurally distinct polypeptides which co-electrophorese with actin.

#### Morphological Appearance of *D. discoideum* Membranes and Membrane Cytoskeletons

The isolated *D. discoideum* membranes appear as a heterogeneous mixture of intact mitochondria and other vesicular

<sup>2</sup> Although relative intensities of individual spots are variable from one iodination to another and absolute mobilities of spots are variable from one map to another, the positions of particular spots, relative to the other spots, are reproducible. See Luna et al. (44) for a discussion of reproducibility with this technique.

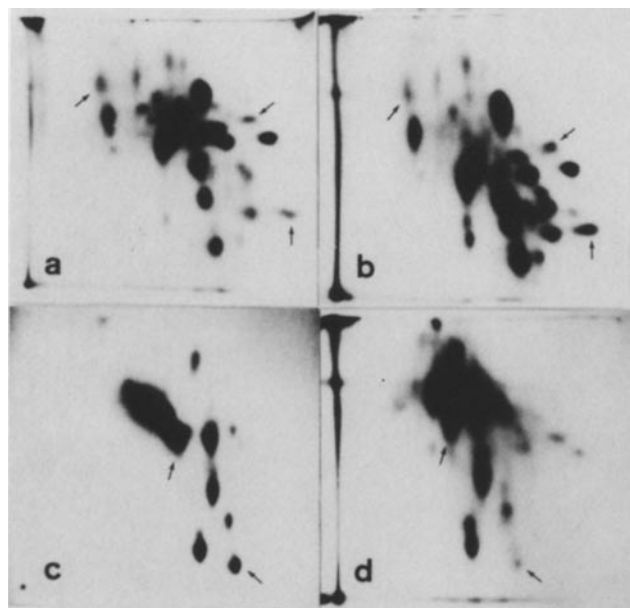


FIGURE 2 Comparison of the  $^{125}\text{I}$ -labeled tryptic peptide maps of (a) cytoplasmic *D. discoideum* myosin, (b) *D. discoideum* membrane 230,000-dalton polypeptide (Fig. 1, band 1.1), (c) cytoplasmic *D. discoideum* actin, and (d) *D. discoideum* membrane 43,000-dalton polypeptide (Fig. 1, band 5). For orientation, selected spots are labeled with arrows.

membranes (Fig. 3a). Lysosomes, nuclei, ribosomes, and rough endoplasmic reticulum appear to be largely absent. At high magnification, the bold profiles of the most intensely stained vesicles (Fig. 3a) are visualized as 7.0-nm-thick trilaminar structures (Fig. 3b) that are typical of *D. discoideum* plasma membranes (28, 49, 62).

The pellet resulting from Triton extraction of isolated *D. discoideum* membranes is enriched in vesicles and linear segments presumably derived from plasma membranes (Fig. 3c). Both forms of boldly profiled membrane retain the characteristic trilaminar appearance of unextracted plasma membranes although the width of the unit membrane is not as regular as that observed for unextracted membranes (Fig. 3b vs. Fig. 3d). The Triton pellet is also enriched in unidentified amorphous material which may represent either poorly preserved actin-containing cytoskeletal structures, or the remnants of a detergent-insoluble matrix from inner mitochondrial membranes (30), or both. In any case, the structures observed in Fig. 3c and d do not contain appreciable phospholipid because at least 95% of the organic solvent-extractable phosphorus is retained in the Triton supernate.

### Actin Cross-linking<sup>3</sup> Induced by *D. discoideum* Membranes

Thus far, the data suggest the existence of one or more membrane-associated protein complexes that are stabilized by, and thus interact with, actin or myosin, or both. To assay for interactions between actin and *D. discoideum* membranes, we use a novel application of low-shear viscometry (Fig. 4a). Our

<sup>3</sup> In this paper, the term "cross-linking" refers to interactions of actin filaments with polypeptides or membranes containing a multiplicity of actin-binding sites. These interactions give rise to anastomosing structures in which actin filaments are bound to one another or "cross-linked."

rationale for this approach is that membranes containing many F-actin-binding sites may cross-link, and thus increase the viscosity of, F-actin in a manner analogous to that observed for cytoplasmic actin-cross-linking proteins (8, 9, 32, 46, 47, 75, 85). We demonstrate the validity of this approach in experiments with erythrocyte membranes (25).

Freshly isolated *D. discoideum* membranes contain appreciable, though variable, amounts of endogenous actin (Fig. 4b, lane 1) and induce only slight increases in the viscosities of actin-containing solutions (Fig. 4a). After extraction with low ionic strength buffers, the membranes lose nearly all of their actin (Fig. 4b, lane 2) and induce large increases in the viscosities of solutions containing actin (Fig. 4a). By analogy to the experiments with erythrocyte membranes (25), we expect that these membrane-induced increases in viscosity are attributable to the interactions between actin and membrane-associated actin-binding components.

To determine the nature of these components, *D. discoideum* membranes are treated with a series of extraction solutions. The removal of most of the major polypeptides with a solution containing KCl and urea (Fig. 4b, lane 3) does not appreciably diminish the ability of the membranes to cross-link actin (Fig. 4a). Therefore, the cross-linking of actin by *D. discoideum* membranes is not readily attributable to the intermediation of a peripheral protein. The destruction of actin cross-linking activity by preincubation of either the low ionic strength-extracted or KCl, urea-extracted membranes at 90°C for 10 min (Fig. 4a) indicates that the actin-binding components are probably proteins. These data suggest that the components responsible for actin cross-linking activity are integral membrane proteins, a suggestion strengthened by the following observations: (a) actin cross-linking activity remains associated with *D. discoideum* membranes even after extraction of the membranes with 0.1 N NaOH; (b) actin cross-linking activity is not observed if either DPPC vesicles or vesicles prepared from *D. discoideum* lipids are added to assay buffer containing actin; and (c) actin cross-linking activity is diminished if the membranes are pretreated with pronase (Fig. 5). The effects of pronase pretreatment (Fig. 5a) cannot be ascribed to residual protease activity because actin remains intact, as judged by SDS polyacrylamide gel electrophoresis, after incubation with proteolyzed membranes (data not shown). If the pronase is not completely inhibited, actin proteolysis is observed on SDS polyacrylamide gels and viscosities of mixtures of actin and pronase-pretreated membranes are lower than viscosities observed for actin alone.

### Actin Cross-linking in the Presence of Triton X-100

The inclusion of 3% Triton during the assay incubation (28°C, 1 h) has no qualitative effect on the actin cross-linking activities of most of the *D. discoideum* membrane preparations assayed in Figs. 4 and 5 (data not shown). However, the inclusion of Triton partially restores the actin cross-linking activity of heat-denatured membranes (Fig. 4a). This restoration of activity is consistent with the idea that the actin-binding components are proteins intimately associated with the bilayer interior. Conversely, when Triton is present during the assay, NaOH-extracted membranes lose most of their actin cross-linking activity (Fig. 5a) and pronase-pretreated membranes lose the rest of their already-scant activity (data not shown). These observations imply that, besides containing actin-binding proteins, the less harshly treated membranes may also

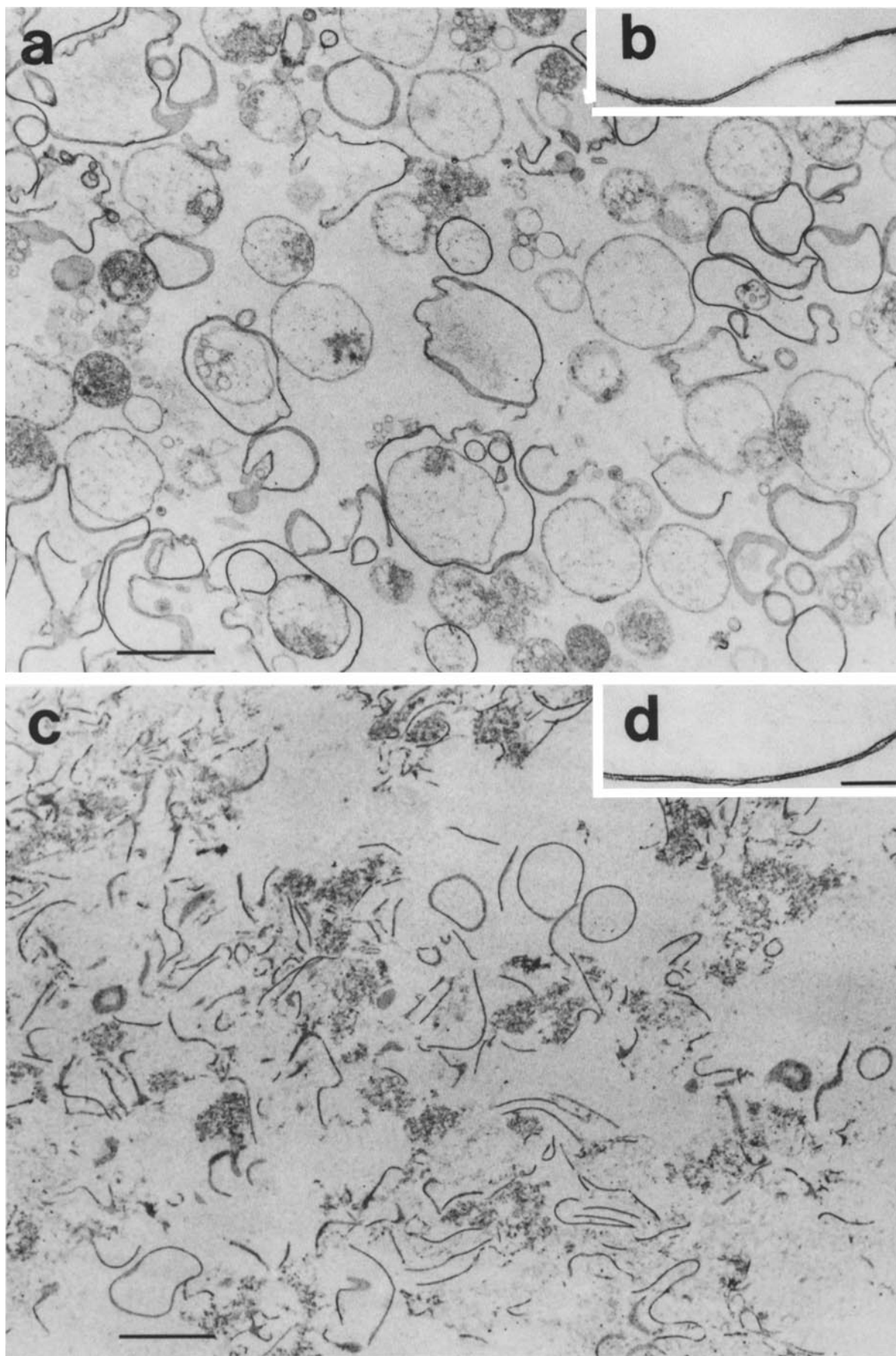


FIGURE 3 Electron micrographs of (a and b) isolated *D. discoideum* membranes and (c and d) the pellet obtained after Triton extraction. (a and c) Bars, 1  $\mu$ m.  $\times$  17,000 (approx.). (b and d) Bars, 100 nm.  $\times$  108,000 (approx.)



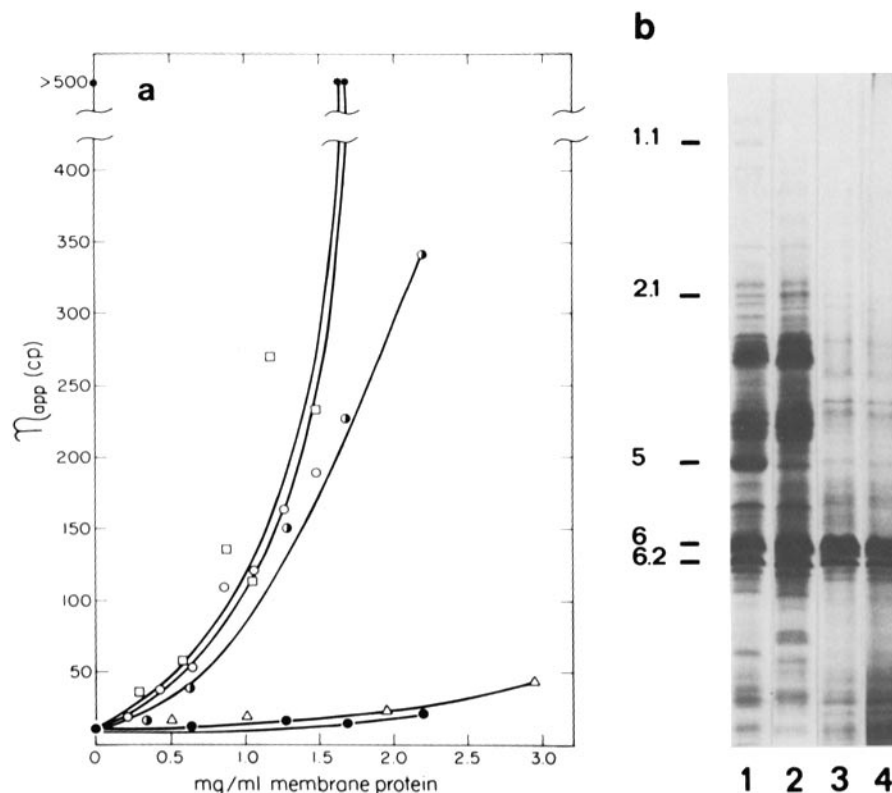


FIGURE 4 (a) Apparent viscosity (centipoise) vs. concentration of membrane protein (mg/ml) for *D. discoideum* membrane preparations mixed with 0.8 mg/ml  $G_i$ -actin and incubated in assay buffer at 28°C for 60 min (actin-polymerizing conditions). ( $\Delta$ ) Isolated *D. discoideum* membranes containing endogenous actomyosin, ( $\square$ ) low ionic strength-extracted membranes, ( $\circ$ ) KCl, urea-extracted membranes, ( $\bullet$ ) KCl, urea-extracted membranes heated with excess crystalline DTT at 90°C for 10 min before assay, and ( $\bullet$ ) heat-pretreated KCl, urea-extracted membranes incubated in assay buffer containing 3% Triton X-100. Viscosities of the other membrane-actin mixtures were relatively independent of the presence of Triton during the assay. In the absence of actin, all *D. discoideum* membrane preparations had viscosities of 1–2 cp. (b) 6–16% SDS polyacrylamide gels of the *D. discoideum* membrane preparations used in Fig. 4 a. (1) Isolated *D. discoideum* membranes containing endogenous actin (band 5) and myosin (band 1.1), (2) low ionic strength-extracted membranes, (3) KCl, urea-extracted membranes, and (4) heat-pretreated KCl, urea-extracted membranes. About 40  $\mu$ g of membrane protein was loaded on each lane. The lanes are from a single gel.

contain components responsible for conferring multivalency on the actin-binding proteins in the presence of Triton. These putative components, which may be protein or unextracted lipid, or both, seem to be absent or inactive in the NaOH- and pronase-treated membranes.

### Assay Conditions

Increasing the free calcium ion concentration from  $\sim 3 \times 10^{-8}$  M (free calcium ion concentration of assay buffer) to  $\sim 5 \times 10^{-6}$  M has no effect on the actin cross-linking activities of *D. discoideum* membranes in some experiments and a slight inhibitory effect in others (data not shown). Increasing the KCl concentration to 500 mM or incubating at 0°C instead of 28°C lowers the viscosities of all actin-containing solutions, but membrane-actin mixtures still exhibit large increases in viscosity as compared with solutions containing actin only (data not shown).

### *D. discoideum* Membranes Cross-link F-Actin

Fig. 6 illustrates that low ionic strength-extracted *D. discoideum* membranes cross-link both preformed F-actin ( $F_i$ -actin) and actin initially added as G-actin but incubated under polymerizing conditions ( $G_i$ -actin). Although the viscosities of suspensions of membranes plus  $F_i$ -actin are less than the viscosities of equivalent amounts of membranes plus  $G_i$ -actin,

these differences are probably attributable to the fact that solutions of freshly polymerized actin are not completely thixotropic. The viscosities of these solutions drop upon the first mechanical disruption of the solution (Fig. 6a and reference 8), with subsequent disruptions resulting in little, if any, additional decrease in viscosity (Fig. 6b and reference 8). Whereas  $F_i$ -actin is mechanically disturbed by mixing with membranes and buffer for the viscosity assay,  $G_i$ -actin is mixed with membranes, allowed to polymerize, and measured without disturbance (Fig. 6). Mechanical disruption of preincubated  $G_i$ -actin-membrane mixtures results in viscosities that are approximately the same as viscosities of  $F_i$ -actin-membrane mixtures (Fig. 6a). Viscosities of  $F_i$ -actin-membrane mixtures are relatively independent of further mechanical disruption (Fig. 6b).

### Membrane-induced Actin Cross-linking Reflects Actin Binding to the Membranes

Actin cross-linking induced by extracted *D. discoideum* membranes is paralleled by an increase in actin binding to the membranes, for both  $F_i$ -actin and  $G_i$ -actin (Fig. 7). The amount of actin that pellets with the membranes varies in rough proportion to the actin cross-linking activity of the membranes (compare Figs. 4a, 5a, and 6 with Fig. 7). An exception is *D. discoideum* membranes containing endogenous actin. These



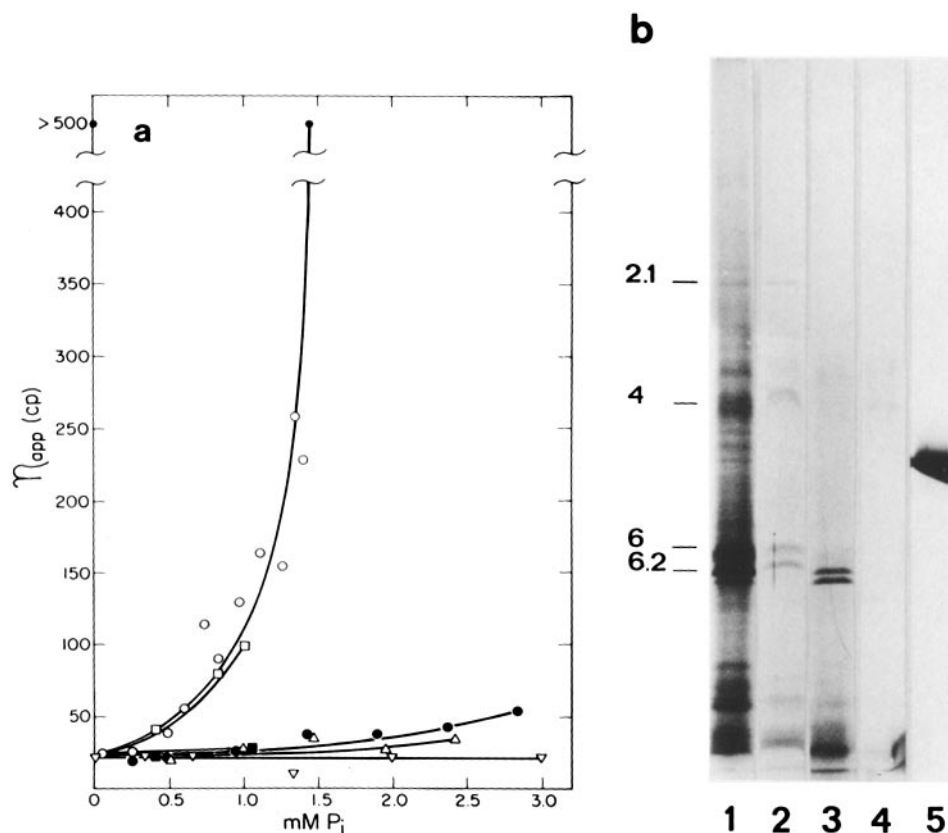


FIGURE 5 (a) Apparent viscosity (centipoise) vs. concentration of phospholipid phosphorus (mM  $P_i$ ) for lipid vesicles and *D. discoideum* membrane preparations mixed with 0.8 mg/ml  $G_i$ -actin and incubated in assay buffer at 28°C for 60 min. ( $\nabla$ ) DPPC vesicles, ( $\Delta$ ) *D. discoideum* lipid vesicles, ( $\bullet$ ) protease-pretreated KCl, urea-extracted membranes, ( $\circ$ ) KCl, urea-extracted membranes incubated and centrifuged as a control for the protease-pretreated membranes, ( $\square$ ) NaOH-extracted membranes and ( $\blacksquare$ ) NaOH-extracted membranes incubated in assay buffer containing 3% Triton X-100. Viscosities of the other membrane-actin mixtures were relatively independent of the presence of Triton during the assay. In the absence of actin, all vesicle and membrane preparations had viscosities of 1–2 cp. (b) 6–16% SDS polyacrylamide gels of the *D. discoideum* membrane preparations used in Fig. 5 a. (1) KCl, urea-extracted membranes incubated and centrifuged as a control for the protease-pretreated membranes, (2) NaOH-extracted membranes, (3) protease-pretreated KCl, urea-extracted membranes, (4) *D. discoideum* lipid vesicles, and (5) 11  $\mu$ g of rabbit skeletal muscle actin. About 0.07  $\mu$ mol of phospholipid phosphorus was loaded on each lane containing membrane. The lanes are from a single gel.

membranes are not very active in cross-linking actin although they induce the pelleting of exogenous  $G_i$ -actin under polymerizing conditions (Fig. 7 a, lane 2), presumably by the addition of G-actin to preexisting membrane-associated actin filaments. This phenomenon is similar to that documented for the binding of G-actin to human erythrocyte membranes (15, 25).

Although heat denaturation or proteolysis of KCl, urea-extracted membranes decreases both actin cross-linking (Figs. 4 a and 5 a) and actin-binding (Fig. 7) activities, these pretreated membranes, which do not effectively cross-link actin, still induce the pelleting of more actin than is sedimented in the absence of membranes (Fig. 7 a and b, lanes 1, 5, and 7). This increased sedimentability of actin may be caused by nonspecific binding of actin to the membranes, or it may be caused by a specific binding of actin which does not generate increases in viscosities of actin-containing solutions. (See reference 25 for a discussion of the ways in which membranes may bind to actin filaments without cross-linking them.)

#### Additional Subcellular Fractionation of Actin Cross-linking Activity

Further purification of low ionic strength-extracted *D. discoideum* membranes on a Renografin density gradient as de-

scribed by McMahon et al. (49) fractionates these membranes into two turbid bands (49, 87). The lower, yellow band is enriched in mitochondria and depleted of vesiculated plasma membranes as monitored by electron microscopy (Fig. 8 a and b, and references 49 and 87) and by assays of the mitochondrial marker, cytochrome *c* oxidase, and the presumed plasma membrane marker, alkaline phosphatase (data not shown). The upper, white band is enriched in plasma membrane fragments and depleted of mitochondria (Fig. 8 a and c, and references 49 and 87).

Most of the actin cross-linking activity associated with the low ionic strength-extracted membranes is recovered from the Renografin gradient in the upper band (Fig. 9 a) which contains some of the same polypeptides (Fig. 9 b, lane 3) as the KCl, urea-extracted and NaOH-extracted membranes (Fig. 5 b, lanes 1 and 2). The lower band has little actin cross-linking activity, suggesting that mitochondria are not responsible for the observed activity of *D. discoideum* membranes.

When intact *D. discoideum* amoebae are coated with Con A and then extracted with 0.2% Triton X-100, portions of the *D. discoideum* plasma membranes are selectively stabilized against solubilization (16, 55). Subsequent fractionation on a sucrose gradient results in a turbid band containing highly purified sheets of plasma membrane (Fig. 10 and reference 16). These

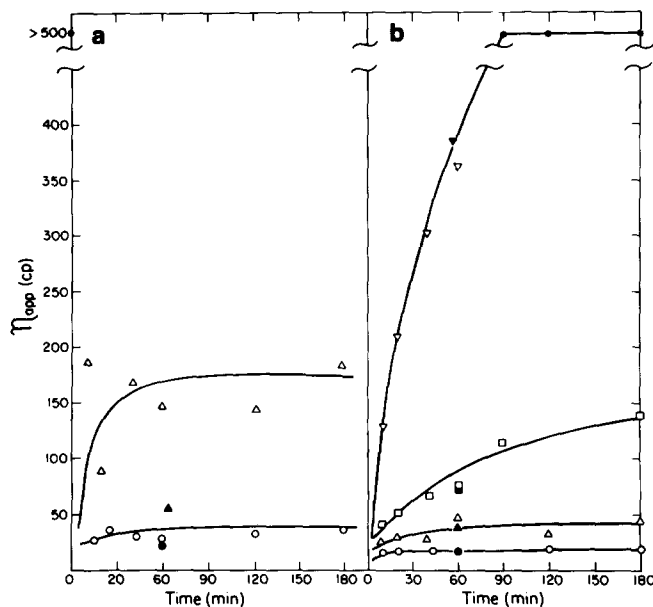


FIGURE 6 Increases in viscosity with time of (a) G-actin or (b) F-actin incubated in assay buffer at 28°C (actin-polymerizing conditions) with and without low ionic strength-extracted *D. discoideum* membranes. (○) Actin alone, (Δ) actin and membranes (0.5 mg/ml membrane protein), (□) actin and membranes (1.0 mg/ml membrane protein), (∇) actin and membranes (2.0 mg/ml membrane protein). Filled symbols correlate with the open symbols and indicate mixtures of actin plus membranes or actin alone that were preincubated for 60 min at 28°C in a test tube and then vortexed and incubated for 60 min in the micropipettes before measuring the viscosity.

Con A-Triton plasma membranes cross-link actin under conditions analogous to those observed for other *D. discoideum* membrane preparations (Fig. 10b). Freshly isolated Con A-Triton plasma membranes contain endogenous actin (Fig. 10c, lane 2) and induce only slight increases in viscosity over that of actin alone (Fig. 10b). The selective extraction of Con A, actin, and myosin (Fig. 10c, lane 3) results in a membrane preparation with the capacity to cross-link actin (Fig. 10b).

Most of the darkly staining bands from Con A-Triton plasma membranes (Fig. 10c, lane 2) comigrate on SDS polyacrylamide gels with prominent bands on gels of other *D. discoideum* membrane preparations (Figs. 1, 4b, and 9b). Discrepancies between the gel pattern of Con A-Triton plasma membranes and the gel patterns of the other membrane fractions are presumably caused by one or more of the following factors: (a) differential contamination of plasma membranes by proteins from other organelles, (b) specific redistribution of membrane proteins into or out of the Con A-stabilized regions of the plasma membrane, (c) differential elution of proteins during purification, and (d) different degrees of proteolysis. While the Con A-Triton plasma membranes are freer of contaminating organelles than are the other membrane preparations, preliminary two-dimensional tryptic peptide mapping data show that Con A-Triton plasma membranes are subject to significant proteolysis (data not shown), possibly because of the lysis of digestive vacuoles during Triton extraction. Although these findings suggest caution if using Con A-Triton plasma membranes as a source of proteins of independently unsubstantiated molecular weight, the Con A-Triton preparative procedure is an excellent method for isolating highly purified regions of *D. discoideum* plasma membranes.

## DISCUSSION

This study presents evidence for the existence of a *D. discoideum* membrane cytoskeleton, that is, a group of lipids and proteins that remain associated after Triton X-100 extraction and that include cytoskeletal elements and any associated intrinsic or extrinsic membrane polypeptides. Our results show that the *D. discoideum* membrane cytoskeleton contains actin and myosin (Fig. 2) and is dissociated in the presence of MgATP (Fig. 1b), a reagent that dissociates actin and myosin. This result suggests that actin and myosin stabilize the *D. discoideum* membrane cytoskeleton and implies an association

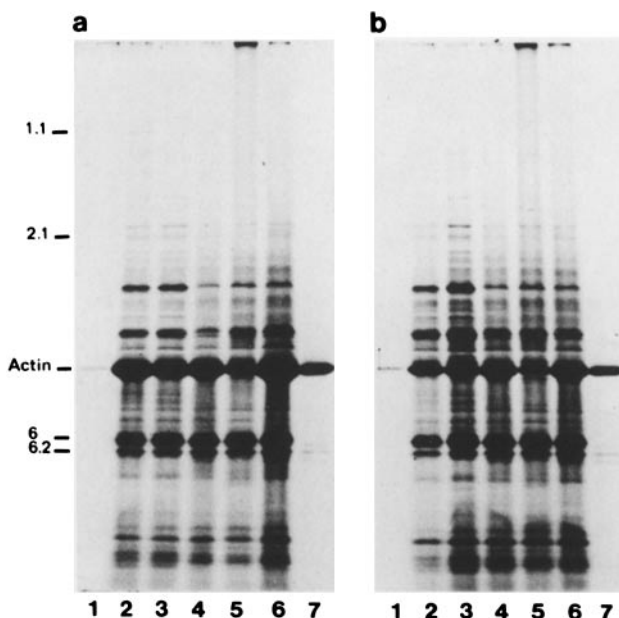


FIGURE 7 6–16% SDS polyacrylamide gels of pellets obtained from the centrifugation of (a) G-actin and (b) F-actin incubated in assay buffer with and without *D. discoideum* membrane preparations. (1) Actin alone, (2) actin plus membranes containing endogenous actin, (3) actin plus low ionic strength-extracted membranes, (4) actin plus KCl, urea-extracted membranes, (5) actin plus heat-denatured KCl, urea-extracted membranes, (6) actin plus KCl, urea-extracted membranes incubated and centrifuged as a control for pronase-pretreated membranes, and (7) actin plus pronase-pretreated membranes. Gels of corresponding membrane preparations before the addition of exogenous actin are presented in Fig. 4b (lanes 1–4) and Fig. 5b (lanes 1 and 3). Rabbit muscle G-actin or F-actin (polymerized at 5.4 mg/ml in 100 mM KCl, 2.0 mM MgATP, 20 mM PIPES, pH 6.8, 1 h, 28°C) was mixed and incubated with or without membranes in assay buffer. Final protein concentrations of actin and membranes were 0.8 and 0.5 mg/ml, respectively—except for the pronase-pretreated membranes which were normalized to the unproteolyzed membranes on the basis of phospholipid phosphorus concentrations. Immediately after mixing, 0.2 ml of each sample was layered over 0.15 ml of 20% sucrose, 20 mM sodium phosphate, 0.01% sodium azide, pH 6.8, in a 0.4 ml polyethylene microfuge tube so that an air space remained between the sample and the sucrose shelf. The microfuge tubes were then incubated at 28°C for 60 min and centrifuged at 23,500 g for 70 min at 10°C in a Sorvall HB-4 rotor. (If a fixed angle rotor was used, the pellets collected along the sides of the tubes instead of at the bottom.) Supernates and sucrose shelves were discarded, pellets were resuspended to 50  $\mu$ l with 20 mM sodium phosphate, pH 6.8, and 12.5  $\mu$ l of 5X-concentrated electrophoresis sample buffer were added. Samples were immediately solubilized by heating at 70°C for 5–10 min. Ten  $\mu$ l of each sample were loaded onto the gel.

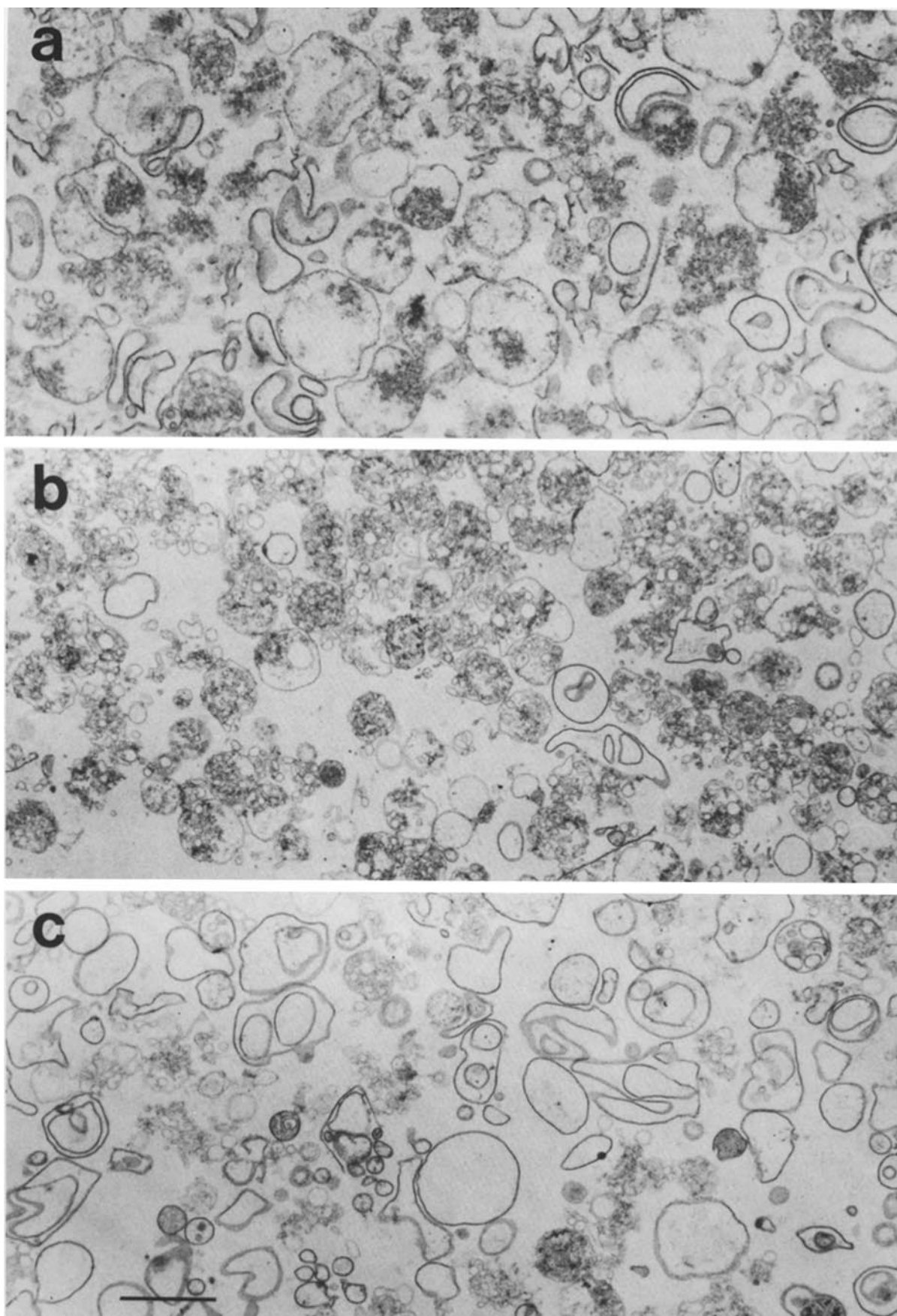


FIGURE 8 Electron micrographs of low ionic strength-extracted *D. discoideum* membranes before and after fractionation on a Renografin density gradient (49, 87). (a) Membranes before fractionation, (b) the lower, yellow band, and (c) the upper, white band recovered from this gradient. Bar, 1  $\mu$ m.  $\times$  17,000 (approx.).

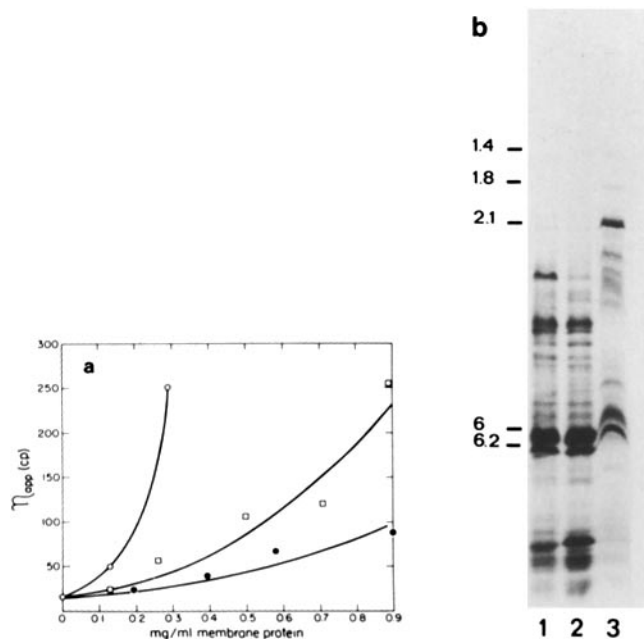


FIGURE 9 (a) Apparent viscosity (centipoise) vs. concentration of membrane protein (mg/ml) for the *D. discoideum* membrane preparations visualized in Fig. 8. Membranes were mixed with 0.8 mg/ml G<sub>i</sub>-actin in assay buffer and incubated at 28°C for 60 min. (□) low ionic strength-extracted membranes before fractionation (Fig. 8a), (●) the lower, yellow band (Fig. 8b), and (○) the upper, white band from the Renografin gradient (Fig. 8c). (b) 6–16% SDS polyacrylamide gels of the *D. discoideum* membrane preparations visualized in Fig. 8. (1) Low ionic strength-extracted membranes before fractionation (Fig. 8a), (2) the lower, yellow band (Fig. 8b), and (3) the upper, white band from the Renografin gradient (Fig. 8c). About 25  $\mu$ g of protein was loaded onto each lane.

between myosin and the membrane cytoskeleton, or between actin and the membrane cytoskeleton, or both.

Using falling ball viscometry (29, 46) to monitor membrane-induced actin cross-linking, we have detected and partially characterized an interaction between actin and one or more membrane-associated actin-binding components. Because actin cross-linking activity is diminished or abolished by heat- or protease-pretreatment of previously active membranes and because lipid vesicles do not appreciably cross-link actin, we conclude that these actin-binding components are proteins. These proteins are probably integral because the membranes retain actin cross-linking activity after being extracted with 1 M KCl, 3.5 M urea or with 0.1 M NaOH (Figs. 4a and 5a). Considering the magnitude of the effects induced by *D. discoideum* membranes in solutions of polymerizing or preformed F-actin, we feel that a reasonable explanation for the membrane-induced actin cross-linking is a lateral association of actin filaments with membrane proteins. Precedents for lateral associations between actin filaments and membrane-associated proteins include the electron microscope observations that cross-bridges exist between microvillar membranes and the sides of actin bundles (50, 51) and that erythrocyte spectrin<sup>4</sup> and spectrin-band 4.1 complexes (83, and footnote 4) bind along the lengths of actin filaments. Finally, actin filaments

<sup>4</sup> Cohen, C. M., J. M. Tyler, and D. Branton. 1980. Spectrin-actin associations studied by electron microscopy of shadowed preparations. *Cell* 21:875–883.

running close and parallel to membranes have been observed in myoblasts (65) and in myoid cells of testis (82).

We do not exclude the possibility of interactions between actin and *D. discoideum* membranes other than those monitored by actin cross-linking. As discussed in the accompanying paper (25), the attachment of actin filaments through the binding of only one end to membrane-associated sites is unlikely to generate viscosities of the magnitude documented here. However, such “end-on” binding of actin to membranes may be responsible for much of the total amount of actin bound to *D. discoideum* membranes. In fact, end-on binding of actin filaments has been observed for Con A-capped *D. discoideum* membranes (16) as well as for actin-membrane interactions in nonmuscle cells (5, 10, 21, 51, 53, 56, 61) and for the actin-Z-disk interaction in muscle (35, 36).

### Limitations of Membrane-induced Actin Cross-linking

The major limitation of using selective membrane extractions and membrane-induced actin cross-linking as a technique to identify membrane-associated actin-binding components is the lack of quantitation inherent in this approach. First, the viscosities reported here are only approximate and not absolute because actin-containing solutions are not Newtonian fluids (48). Consequently, large variations in apparent viscosity may be caused by small changes in cross-linker (membrane) concentration. Second, the extraction procedures, independent of any polypeptides extracted, may directly affect the cross-linking abilities of membranes. For instance, an extraction procedure may increase the affinity of an actin-binding protein for its substrate by denaturing other proteins that regulate this interaction. Third, extraction procedures may indirectly affect the cross-linking abilities of membranes. In our study of actin cross-linking by erythrocyte membranes, we find that only inside-out vesicles are capable of cross-linking actin (25). An extraction procedure that alters the ratio of inside-out to right-side-out vesicles is thus likely to affect the actin cross-linking activity of the membranes. Also, for a given membrane protein concentration, the effectiveness of the membranes in cross-linking actin will depend upon the diameter of the vesicles. A smaller average vesicle diameter (assuming that all vesicles are still multivalent with respect to actin-binding sites) means a larger number of “cross-linkers” per milligram of protein. Thus, an extraction procedure that increases vesiculation may increase the ability of the membranes to cross-link actin even though the extraction has partially removed the actin-binding protein. These considerations mandate caution in the interpretation of results obtained from actin cross-linking assays. If these limitations are appreciated, falling ball viscometry is a useful semiquantitative technique for studying the interactions of actin with small quantities of membranes.

### Intracellular Distribution of Actin Cross-linking Activity

Because actin cross-linking activity is associated with both the plasma membrane-enriched fraction from a Renografin gradient (Figs. 8 and 9a) and with plasma membrane domains stabilized by Con A (Fig. 10), some of the actin cross-linking activity described here is probably associated with some portions of the *D. discoideum* plasma membrane. But the actin cross-linking proteins may not be limited to the *D. discoideum*

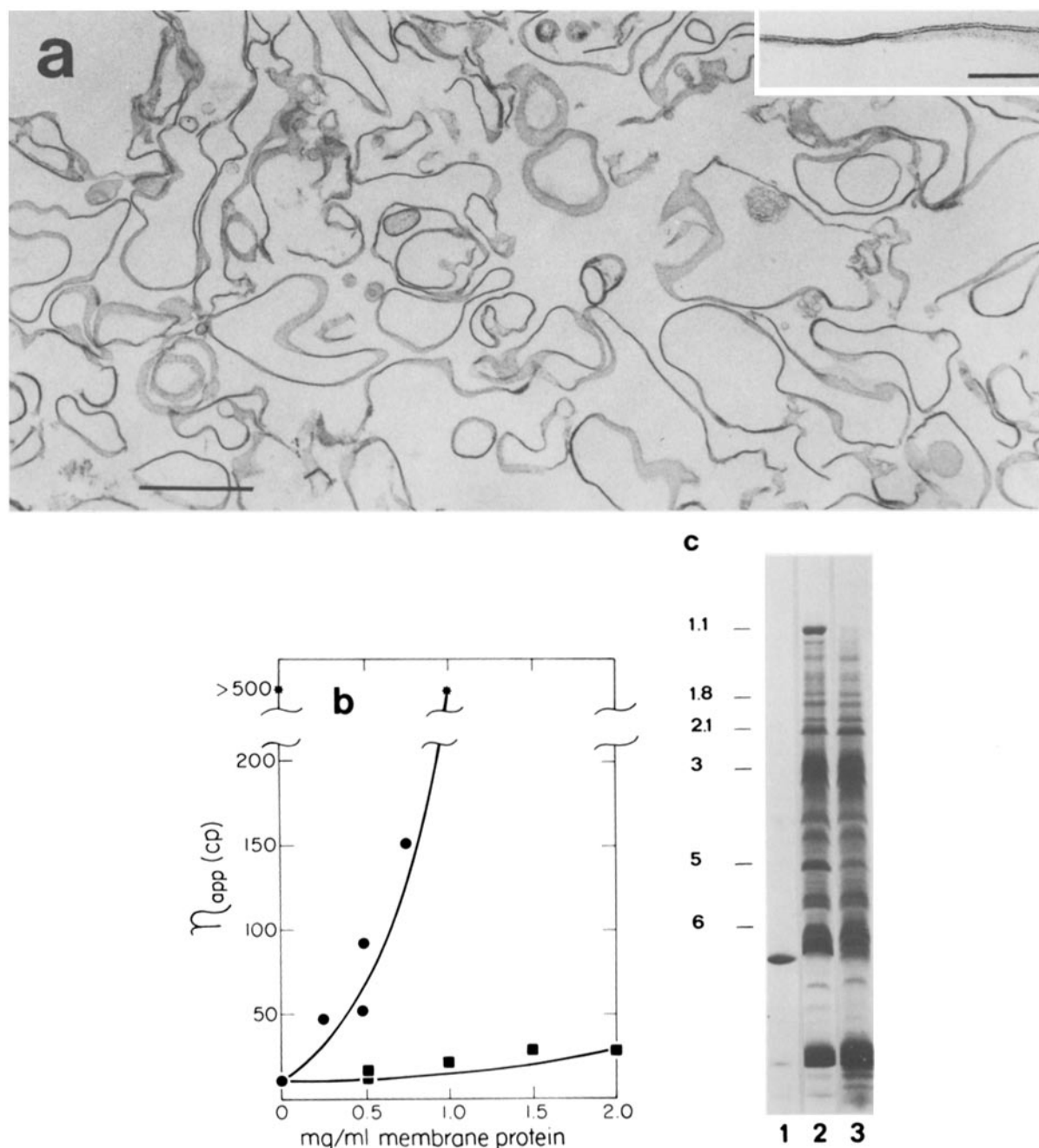


FIGURE 10 (a) Electron micrographs of *D. discoideum* plasma membranes obtained after extraction with 0.2% Triton X-100 from amoebae preincubated at room temperature for 15 min with 60  $\mu\text{g}/\text{ml}$  Con A (Con A-Triton plasma membranes). Purification was performed as described by Condeelis (16). Bar, 1  $\mu\text{m}$ .  $\times 17,000$  (approx.). Inset: Bar, 100 nm.  $\times 108,000$  (approx.). (b) Apparent viscosity (centipoise) vs. concentration of membrane protein (mg/ml) for 0.8 mg/ml  $G_i$ -actin mixed and incubated with (■) the Con A-Triton plasma membranes visualized in Fig. 10 a and (●) Con A-Triton plasma membranes after the partial removal of Con A with 1.0 M  $\alpha$ -methylmannoside and the removal of actomyosin by low ionic strength extraction as described in Materials and Methods. (c) 6–16% SDS polyacrylamide gels of (1) 5  $\mu\text{g}$  Con A, (2) Con A-Triton plasma membranes (62  $\mu\text{g}$  protein), and (3) Con A-Triton plasma membranes after removal of Con A and actomyosin (53  $\mu\text{g}$  protein). The lanes are from a single gel. Numbered bands appear to correspond with those designated by the numerical nomenclature of Fig. 1.

plasma membrane. The upper band from the Renografin gradient is enriched in plasma membranes as monitored by electron microscopy (Fig. 8 and reference 49). This band is also enriched in 5'-nucleotidase (49, 87) and in alkaline phosphatase (data not shown), enzymes used by many researchers as markers for *D. discoideum* plasma membranes (18, 26, 28, 49, 55, 62, 66, 87). However, a recent cytochemical investigation

(60) demonstrates the presence of both of these presumed plasma membrane markers in the large contractile vacuoles of *D. discoideum* rather than at the plasma membrane. If correct, this observation suggests that most *D. discoideum* plasma membrane preparations are contaminated with contractile vacuoles. Thus, contractile vacuoles may be responsible for some of the observed actin cross-linking activity. Also, since *D. discoideum*

plasma membranes are in temporal and spatial equilibrium with the membranes of pinocytotic vesicles (80), actin cross-linking activity may be associated with these or other intracellular organelles.

Actin cross-linking activity that is localized at the plasma membrane is not necessarily uniformly distributed over the surface of this membrane. In fact, electron micrographs of Triton-extracted *D. discoideum* membranes (Fig. 3c) visualize many short, linear segments of material that are apparently derived from the plasma membrane. The appearance of these membranous segments is reminiscent of the electron microscope appearance of specialized, protein-rich regions derived from other membranes, such as desmosomes (20, 67) and gap junctions (27). Analogous specialized regions have not yet been documented in *D. discoideum*, but, even in the absence of exogenous stabilizing lectins such as Con A, parts of the *D. discoideum* plasma membrane are relatively resistant to solubilization by Triton X-100.<sup>5</sup>

Although no evidence directly associates actin cross-linking activity with any of the *D. discoideum* membrane polypeptides, it is interesting that three major Coomassie Blue-staining bands, designated as 2.1, 6, and 6.2 (Fig. 1), are apparently present in each membrane preparation capable of cross-linking actin (Figs. 4, 5, 9, and 10). Two of these bands (6 and 6.2) are especially prominent, but further investigation is required before functions can be assigned to these, or other, polypeptides associated with the *D. discoideum* membrane cytoskeleton.

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